The role of 2-methyl-6-phytylbenzoquinone methyltransferase in determining tocopherol composition in *Synechocystis* sp. PCC6803

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Abstract A putative 2-methyl-6-phytylbenzoquinone (MPBQ) methyltransferase gene, SLL0418, was identified from the *Synechocystis* PCC6803 genome based on its homology to previously characterized γ-tocopherol methyltransferases. Genetic and biochemical evidence confirmed open reading frame (ORF) SLL0418 encodes a MPBQ methyltransferase. An SLL0418 partial knockout mutant accumulated β-tocopherol with no effect in the overall tocopherol content of the cell. In vitro assays of the SLL0418 gene expressed in *Escherichia coli* showed the enzyme efficiently catalyzes methylation of ring carbon 3 of MPBQ. In addition, the enzyme also catalyzes the methylation of ring carbon 3 of 2-methyl-6-solanylbenzoquinol in the terminal step of plastoquinone biosynthesis.

Key words: Tocopherol; Vitamin E; Methyltransferase; Plastoquinone; *Synechocystis*

1. Introduction

Tocopherols are a class of compounds that function as lipid soluble antioxidants that are extremely potent quenchers of singlet oxygen and free radical species [1]. Four different tocopherols, α-, β-, γ-, and δ-tocopherol, are synthesized in nature that differ from one another based on the number of methyl groups and the position of these methyl groups on the chromanol head group. Although each of these different forms of tocopherol function as vitamin E in human and animal diets, the relative vitamin E activity associated with each differs significantly. The fully methylated form of tocopherol, α-tocopherol, is by far the most potent form of vitamin E [2] with β-, γ-, and δ-tocopherols having 40, 10, and 1%, respectively, of the vitamin E activity of α-tocopherol [3]. Interestingly, this activity trend is reversed when considering the in vitro antioxidant potential of tocopherols such that δ-tocopherol is the most potent antioxidant followed by γ- and β-tocopherol, and finally α-tocopherol [3].

In photosynthetic organisms, the tocopherol composition of a given cell or tissue is determined by the activity of three enzymes (Fig. 1), 2-methyl-6-phytylbenzoquinone (MPBQ) methyltransferase, tocopherol cyclase, and γ-tocopherol methyltransferase (γ-TMT). The MPBQ methyltransferase methylates the first prenylquinone intermediate in tocopherol biosynthesis, MPBQ, to form 2,3-dimethyl-6-phytylbenzoquinone (DMPBQ). DMPBQ can then be cyclized by the tocopherol cyclase to form γ-tocopherol, which is then methylated by γ-TMT in a terminal reaction to form α-tocopherol. In addition to the primary reactions described above, tocopherol cyclase and γ-TMT can also participate in an alternative biosynthetic scheme leading to the synthesis of β-tocopherol. Specifically, tocopherol cyclase can cyclize MPBQ to δ-tocopherol [4], which can subsequently be converted to β-tocopherol by γ-TMT [5]. Like α-tocopherol, β-tocopherol is thought to be a terminal product since no methyltransferase activity has yet been identified that can convert β-tocopherol to α-tocopherol. The ability to proceed via this alternative pathway requires the absence, or limiting levels of the MPBQ methyltransferase. MPBQ methyltransferase appears to compete with the tocopherol cyclase for MPBQ and is instrumental in directing pathway flux towards α-tocopherol, the primary tocopherol product in photosynthetic cells/organs.

In this paper, we report the functional identification and characterization of MPBQ methyltransferase from the cyanobacterium *Synechocystis* PCC6803. Furthermore, we demonstrated that, in addition to methylating MPBQ, the enzyme is also able to catalyze methylation at the same position on 2-methyl-6-solanasylbenzoquinol (the analogous intermediate in plastoquinone synthesis) and as such plays a dual role in the final methylation step of plastoquinone biosynthesis.

2. Materials and methods

2.1. Generation of SLL0418 mutant in Synechocystis

The SLL0418 DNA sequence was amplified using *Pfu* Taq polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's specifications and *Synechocystis* PCC6803 genomic DNA that was isolated as described by Williams [6]. PCR primers (SLL0418F = 5′-CATATGCCCGAGTATTTGCTTCTG-3′, SLL0418R = 5′-TTTAAGCTTGAGTGGCGTTTTTTC-3′) were designed from the SLL0418 DNA sequence at Cyanobase (www.kazusa.or.jp/cyanobase/about.html). The amplified SLL0418 open reading frame (ORF) was subcloned into the *Eco*RV site in pBluescript KS II (Stratagene), resulting in a plasmid designated as pSLL0418-1. The gene replacement construct, pSLL0418:Kan^R, was prepared by subcloning the kanamycin resistance gene from pUC4K into a blunted *Nco*I site in pSLL0418-1. pSLL0418:Kan^R was transformed into *Synechocystis* PCC6803 as described by Williams [6] and transformants were selected for on BG-11 medium [7] containing 15 mM glucose and 15 mg/l kanamycin. The mutant line was designated SLL0418:Kan^R. All cultures were grown under continuous light at 26°C. Kanamycin resistant transformants were carried through five passages on selection medium.

2.2. Expression of the SLL0418 gene product in Escherichia coli

The *Synechocystis* SLL0418 coding sequence was subcloned in

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frame into the *E. coli* expression vector, pET30B (Novagen, Madison, WI, USA) to generate pET0418 and transformed into the *E. coli* strain BL21(DE3)pLysS. Cells containing pET0418 or the empty vector were grown to an optical density of 0.6 at 600 nm, protein expression induced with 1 mM isopropylthio-β-galactoside (IPTG) and growth continued for an additional 3 h at 28°C. Induced cells were harvested by centrifugation and stored at −80°C. The cell pellet was resuspended in 50 mM Tris–HCl pH 8.0, 5 mM DTT, 1 mM PMSF and homogenized by sonication. Triton X-100 was then added to a final concentration of 1% and the mixture incubated on ice for 30 min. The insoluble material was then removed by centrifugation at 40 000×*g* for 30 min and the supernatant was saved for methyltransferase assays.

2.3. Preparation of 2-methyl-6-phytolbenzoquinol and 2-methyl-6-solanylquinol substrates

A mixture of six different isomers of methylphytylbenzoquinone was synthesized as described by Henry et al. [8]. 250 μg of the mixed isomer preparation was suspended in ethylether and oxidized by adding 100 mg of silver oxide and incubating for 2 h at room temperature. The oxidized isomers were dried under nitrogen gas, resuspended in hexane and MPBQ purified by isocratic chromatography on a 7.8×300 mm μPorasil HPLC column (Waters, Milford, MA, USA) using a mobile phase of 999:1 (v:v) hexane:dioxane. To prepare reduced substrate, MPBQ was resuspended in 1 ml of absolute ethanol to which 50 μl of 50 mg/ml sodium borohydride was added. After 5 min, the reaction was stopped by adding 200 μl of 0.1 M acetic acid and incubating for an additional 5 min at room temperature. Reduced MPBQ was extracted by adding 300 μl of water and 1 ml of hexane. The hexane phase was collected, dried under nitrogen and resuspended in 100 μl of ethanol for use in methyltransferase assays. 2-Methyl-6-solanylbenzoquinone (MSBQ) was purified from iris bulbs as described by Penbrook [9] and reduced or oxidized MSBQ for use in assays prepared as described for the MPBQ substrate.

2.4. 2-Methyl-6-phytylbenzoquinol and 2-methyl-6-solanylbenzoquinol methyltransferase assays

100 μl of solubilized *E. coli* extract containing between 300 and 800 μg protein [10] was assayed for MPBQ methyltransferase activity in a 1 ml reaction containing 50 mM Tris pH 8.0, 5 mM DTT, 1.8 μM [^{14}C-methyl]S-adenosylmethionine (56 mCi/mmol), and 1 mM reduced/oxidized MPBQ or MSBQ. Reactions were incubated at 28°C for 30 min and stopped by the addition of 4.5 ml of 2:1 (v:v) chloroform:methanol and 2 ml of 0.9% (w:v) NaCl. The reaction mixture was then partitioned into two phases, the organic phase removed and dried under a stream of nitrogen gas. The resulting residue was then resuspended in hexane and fractionated on 250 μm 60A KF6 silica gel TLC plates (Whatman, Clifton, NJ, USA) with 3:7 (v:v) diethylether:petroleum ether. In the cases where MSBQ was used as a substrate, plastoquinol-9 purified from iris bulbs [9] was used as a TLC standard. The TLC plates were then placed on film for 2 days and the R_f values of the labeled product were determined. The labeled products were then scraped from TLC plates and radioactivity incorporated was determined by scintillation counting.

2.5. GC-MS analysis

The purified radiolabeled product was subjected to GC-MS analyses using a JEOL AX-505H double-focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph. Samples were separated on a 30 m DB-5ms (0.32 mm inner diameter) capillary column (J&W Scientific, Folsom, CA, USA). Helium gas flow was approximately 1 ml/min. The GC temperature program was initiated at 50°C, 10°C per min to 320°C where it was held for 10 min. MS conditions were as follows: interface temperature >200°C, ion source temperature ca. 200°C, electron energy 70 eV, scan rate of the mass spectrometer 1 s/scan over the *m/z* range 45–750.

3. Results

To identify the MPBQ methyltransferase gene, the *Synechocystis* genome database was searched for sequences showing a high degree of amino acid sequence similarity to the previously cloned and characterized *Synechocystis* γ-tocopherol methyltransferase (γ-TMT) [5]. While this search resulted in the identification of several related gene sequences, ORF SLL0418 shared the highest degree of similarity to *Synechocystis* (43% similarity and 29% identity) and *Arabidopsis* (44% similarity and 30% identity) γ-TMT protein sequences (Fig. 2).

Two structural features of the SLL0418 protein were consistent with its identity as a methyltransferase involved in tocopherol biosynthesis. First, alignment of the two γ-TMT and SLL0418 amino acid sequences revealed that the consensus methyltransferase motifs I, II and III were spatially con-

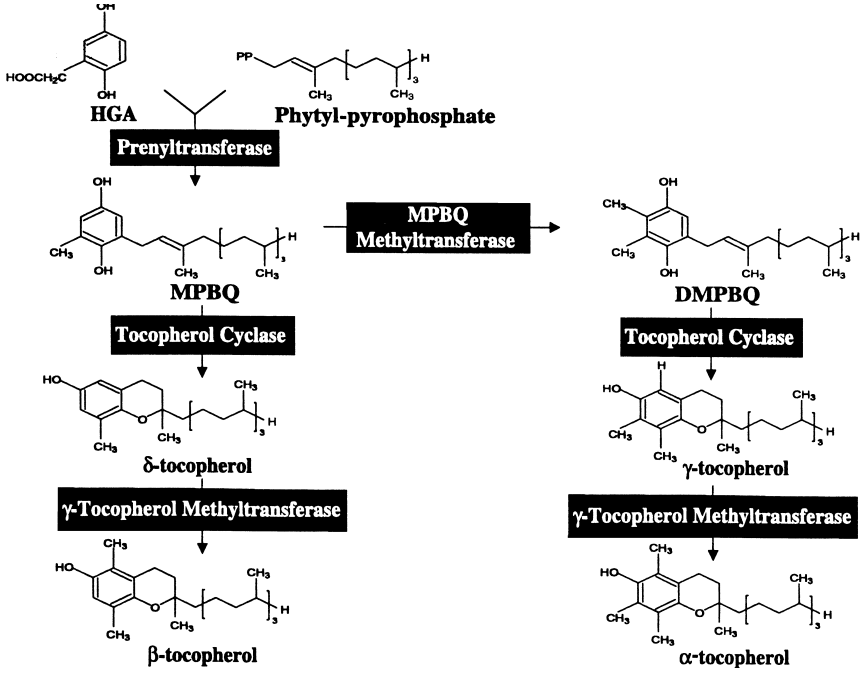

Fig. 1. Tocopherol biosynthetic pathway. HGA, homogentisic acid; MPBQ, 2-methyl-6-phytylbenzoquinone; DMPBQ, 2,3-dimethyl-6-phytylbenzoquinone. For clarity not all substrates and reaction products are shown.

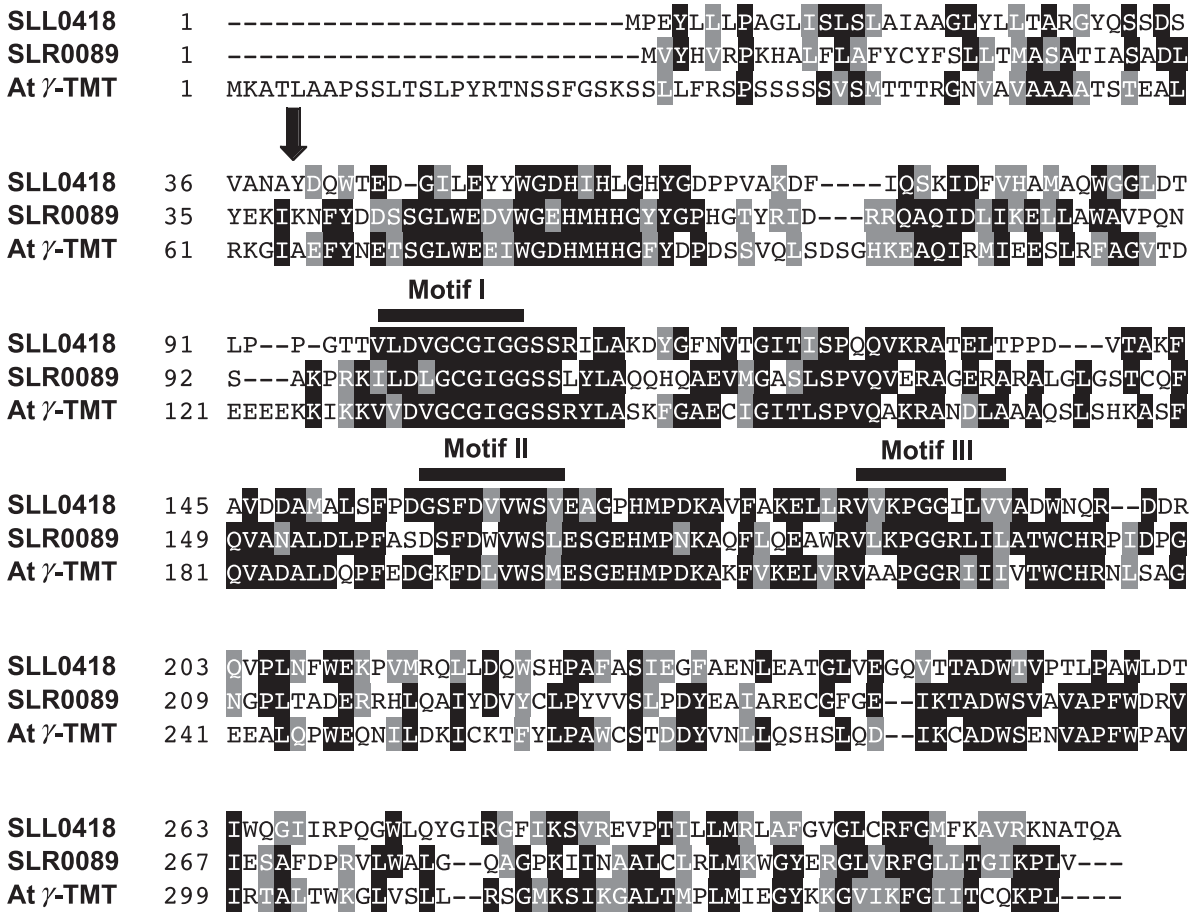

Fig. 2. Protein sequence alignment of SLL0418 and γ-tocopherol methyltransferases. The deduced protein sequence of *Synechocystis* ORF SLL0418 (GenBank accession number BAA18485) was aligned with *Synechocystis* ORF SLR0089 (GenBank accession number BAA105621) and *Arabidopsis* γ-TMT (at γ-TMT) (GenBank accession number AF104220) sequences. Letters highlighted in black and gray represent amino acid residues that are identical or similar, respectively, to those in SLL0418. The black arrow denotes the predicted cleavage site for the bacterial signal sequence. Black bars represent the positions of the indicated methyltransferase motifs.

served among the three proteins [11] (Fig. 2). Second, the SLL0418 protein contains a 39 amino acid N-terminal extension whose structural properties are consistent with signal peptides present in Gram-negative bacteria (Fig. 2). This pre-sequence would target the SLL0418 protein to the plasma membrane [12], which is the presumed site of tocopherol synthesis in *Synechocystis*.

To test the hypothesis that the SLL0418 gene encoded a MPBQ methyltransferase, loss of function experiments were performed in *Synechocystis*. For these studies, a targeted mutation in the SLL0418 gene was created by homologous recombination and the resulting mutant analyzed for changes relative to wild-type tocopherol composition and content. While the tocopherol content of the SLL0418:Kan^R mutant line did not differ significantly from wild-type cells (data not shown), differences in tocopherol composition were observed relative to wild-type (Fig. 3). In addition to the presence of α-tocopherol, SLL0418:Kan^R contained a significant amount of β-tocopherol. This result is consistent with a mutation causing a decrease in MPBQ methyltransferase activity.

To directly demonstrate that the SLL0418 gene product encodes an MPBQ methyltransferase, the full-length SLL0418 ORF was expressed in *E. coli* and the recombinant protein was assayed for MPBQ methyltransferase activity. Protein extracts from *E. coli* cells transformed with the

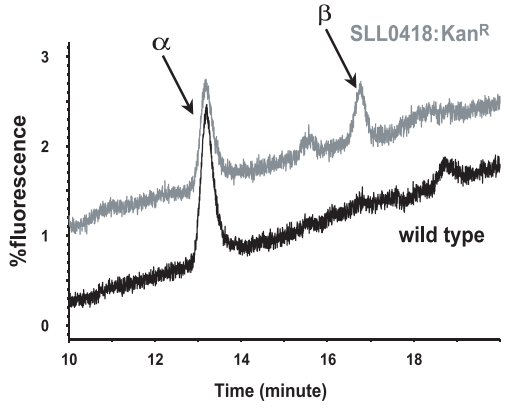

Fig. 3. Tocopherol analysis of wild-type and SLL0418:Kan^R strains of *Synechocystis*. Approximately 200 mg of cells were scraped from 2 weeks old plate cultures of wild-type (black trace) and SLL0418:Kan^R *Synechocystis* (gray trace) cells. Total lipid was extracted from each cell line as described by Bligh and Dyer [14] with 1 mg/ml butylated hydroxytoluene added to the methanol:$CHCl_3$ solution prior to extraction. Tocopherols were fractionated on a Licosorb Si60A 4.6×250 mm HPLC column (Phenomenex, Torrance, CA, USA) using a 20 min linear gradient of 8–18% diisopropylether in hexane at 1 ml/min and detected by fluorescence (excitation λ: 290 nm, emission λ: 325 nm). Individual tocopherol species were identified by comparing their retention time with authentic tocopherol standards (Matreya, Pleasant Gap, PA, USA).

pET0418 expression vector and the pET30A empty vector were assayed for their ability to methylate a synthetically derived MPBQ substrate using [methyl-^{14}C]S-adenosylmethionine. The results of these experiments clearly showed that the SLL0418 protein was capable of methylating reduced or oxidated MPBQ with similar efficiency (specific activity = 395 ± 40 pmol/mg protein/h and 404 ± 20 pmol/mg protein/h for reduced and oxidized MPBQ, respectively). The radiolabeled reduced and oxidized products migrated to the reported R_f for 2,3-dimethyl-6-phytylbenzoquinol and quinone [8], respectively (data not shown).

To confirm the identity of the methylated product from MPBQ, it was purified by normal phase HPLC (Fig. 4) and subjected to GC-MS analysis. For this analysis, the putative 2,3-dimethyl-6-phytylbenzoquinol product was converted to the more stable 2,3-dimethyl-6-phytylbenzoquinone form. The purified, labeled, methylated product had a mass of 414 Da, consistent with the mass of 2,3-dimethyl-6-phytylbenzoquinone (Fig. 4). The recombinant MPBQ methyltransferase enzyme could not methylate other mono-methylated structural isomers, such as 2-methyl-3-phytylplastoquinol and 2-methyl-5-phytylplastoquinol. Additionally, the SLL0418 protein could not methylate δ- and β-tocopherol, two related prenylquinonols that are unmethylated at the identical ring carbon as MPBQ.

Further experiments were performed to determine if, in addition to being involved in tocopherol biosynthesis, SLL0418 also plays a role in plastoquinone biosynthesis. In experiments similar to those described for the MPBQ substrate, assays were performed with *E. coli* expressed SLL0418 protein using MSBQ as a substrate. These experiments showed that the SLL0418 protein catalyzes the methylation of MSBQ to form plastoquinol-9 (specific activity = 461 ± 21 pmol/mg protein/h). The identity of the radiolabeled reaction product was confirmed by co-migration of the radiolabeled product with an authentic plastoquinol-9 standard. Consistent with observations from experiments performed with the MPBQ substrate, recombinant SLL0418 protein was capable of methylating both the oxidized and reduced forms of MSBQ to form plastoquinol-9 or plastoquinone-9, respectively (data not shown).

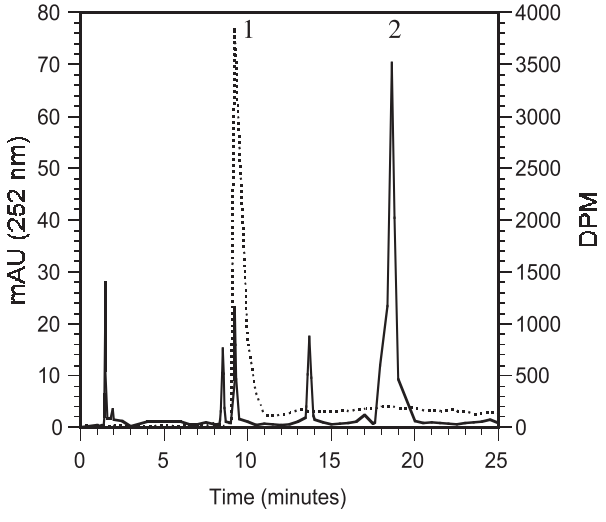

Fig. 4. Purification of MPBQ methyltransferase product. The radiolabeled compound produced using 2-methyl-6-phytylbenzoquinol as substrate was purified on a Licosorb Si60A 4.6×250 mm HPLC column with a 999:1 (v:v) hexane:dioxane mobile phase at a flow rate of 2 ml/min. Fractions were collected in 30 s intervals and subjected to scintillation counting to detect the radiolabeled product. The 252 nm absorbance trace is represented by the solid line trace. Radioactivity is represented by the dashed line trace. Key to compounds: 1. DMPBQ; 2. MPBQ.

4. Discussion

A putative MPBQ methyltransferase gene, ORF SLL0418, was identified in the sequenced genome of *Synechocystis* PCC6803 based on its sequence similarity to the previously characterized γ-TMT enzymes [5]. The SLL0418 gene product is identified as a sterol-C methyltransferase in the *Synechocystis* database, however, based on the work presented in this study we believe this annotation to be incorrect. In the current study, two different approaches were used to demonstrate the identity of this candidate gene as the *Synechocystis* MPBQ methyltransferase. First, loss of function studies in *Synechocystis* were used to show that a directed mutation in the SLL0418 gene resulted in an altered tocopherol composition relative to wild-type cells. Tocopherol analysis of SLL0418:Kan^R showed that in addition to α-tocopherol, which is present as the sole form of tocopherol in wild-type cells, the mutant also contained a substantial amount of β-tocopherol. This was a somewhat unexpected result as though the observed mutant phenotype is still consistent with a decrease in MPBQ methyltransferase activity one would predict that the cellular tocopherol pool of an SLL0418 null mutation would be composed solely of β-tocopherol. The most plausible explanation for this result was that SLL0418:Kan^R was a partial, rather than null, mutant for the SLL0418 gene. This possibility was confirmed by PCR analysis of total genomic DNA isolated from SLL0418:Kan^R that showed both wild-type and mutant copies of the SLL0418 gene were present (data not shown). The dual function of the MPBQ methyltransferase in the tocopherol and plastoquinone pathways could explain why a null mutant for the SLL0418 gene was not isolated. Since plastoquinone is the primary lipid soluble redox carrier in both photosynthetic and respiratory electron transport chains in cyanobacteria, a null mutation affecting plastoquinone synthesis would be difficult to select as it would be anticipated to result in a non-viable or severely compromised phenotype. In maize, high chlorophyll fluorescence mutants (*hcf*103 and *hcf*114), which contain presumed lesions in the MPBQ methyltransferase gene, were shown to be lethal [13].

As a second approach to demonstrate the identity of the SLL0418 protein, we expressed the SLL0418 gene product in *E. coli* and assayed cell extracts for MPBQ methyltransferase activity in vitro using a synthetically derived 2-methyl-6-phytylbenzoquinone (MPBQ) substrate. The recombinant MPBQ methyltransferase was able to methylate MPBQ to form DMPBQ. The combined data of the in vitro assays performed with different substrates suggest that the MPBQ methyltransferase requires substrates with a methyl group in the 2-position and a prenyl group on the 6-position of the benzoquinone head group. Interestingly, the specific activity of the enzyme with the two 'natural' substrates (MPBQ and MSBQ, 20 and 45 carbon prenyl tails, respectively) suggests that prenyl side chain length is not a factor in substrate preference. Although it appeared that the benzoquinone ring is the most important structure in substrate recognition for this

enzyme, the recombinant MPBQ methyltransferase did not discriminate between the reduced and oxidized forms of MPBQ and 2-dimethyl-6-solanylbenzoquinone.

The MPBQ methyltransferase appears to play a more important role in determining tocopherol composition than it does in tocopherol content. This hypothesis is supported by the observation that although SLL0418:Kan^R resulted in an altered tocopherol composition, no significant difference was detected in total tocopherol content. This result indicates that the MPBQ methyltransferase has little influence on overall flux through the tocopherol biosynthetic pathway and may be more important in determining the relative pool size of the different forms of tocopherol.

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